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Alcohol exposure induces chick craniofacial bone defects by negatively affecting cranial neural crest development

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Abstract

Excess alcohol consumption during pregnancy could lead to fetal alcohol syndrome (FAS). However, the molecular mechanism leading to craniofacial abnormality, a feature of FAS, is still poorly understood. The cranial neural crest cells (NCCs) contribute to craniofacial formation, hence NCCs exposed to ethanol was investigated - using chick embryos and *in vitro* explant culture. We demonstrated that exposure to 2% ethanol induced craniofacial defects (including parietal defect) in the developing chick fetus. Immunofluorescent staining revealed that ethanol treatment repressed Ap-2α, Pax7 and HNK-1 expression by cranial NCCs. Using double-immunofluorescent stainings for Ap-2α plus pHIS3 and Ap-2α plus c-Caspase3, we showed that ethanol treatment inhibited cranial NCC cell proliferation and increased apoptosis. Moreover, ethanol treatment of the dorsal neuroepithelium increased Laminin, N-Cadherin and Cadherin 6B expression while Cadherin 7 expression was repressed. *In situ* hybridization also showed that ethanol up-regulated Cadherin 6B expression but down-regulated slug, Msx1, FoxD3 and BMP4 expression. In summary, our experimental results revealed that ethanol treatment interferes with the production of cranial NCCs by affecting the cell proliferation and apoptosis of these cells. In addition, it affected the delamination, epithelial-mesenchymal transition (EMT) and cell migration of cranial NCCs, which may have contributed to the development of the craniofacial defects.

Key words: Alcohol, cranial neural crest cells, delamination, migration, EMT, cell apoptosis.

1. Introduction

The public is now paying more attention to alcohol consumption during pregnancy because alcohol is a potential teratogen and may negatively affect embryo development. McGill el al. have reported that alcohol exposure during the fetal period could increase immune susceptibility to influenza virus and related secondary

respiratory infections (McGill et al., 2009). Ethanol exposure induces malformation in many other tissues and organs -for example, ethanol could disrupt angiogenesis and neurogenesis during embryonic brain development (Bake et al., 2012). In human, excess alcohol consumption during pregnancy can cause collective tissue damage know as fetal alcohol syndrome (FAS) and fetal alcohol spectrum disorders (FASD), The damage includes brain, craniofacial, cardiovascular and limb defects (Abel, 1984, 1995; Jones et al., 1973; Oza et al., 2016). Besides developmental defects, person with FAS also expect a shorter life expectancy (Thanh and Jonsson, 2016). Although the overall cause of FAS is known and preventable, little is known about how craniofacial dysplasia developed in FAS – especially the cellular and molecular mechanisms. In the embryo, the craniofacial bones are derived from cranial neural crest cells (NCCs). Hence, we investigated how ethanol effected the cranial NCCs during development to elucidate the pathogenic mechanisms of ethanol induced craniofacial dysplasia.

Neural crest cells are specified at the border of the neural plate and the non-neural ectoderm after gastrulation, and then arise uniformly at the dorsolateral edge of the closing neural tube during early embryogenesis. These cells successively undergo the developmental process of induction, delamination, EMT, migration and differentiation to eventually give rise to cellular components of many systems in vertebrates (Hall, 2008). The induction of the neural crest occurs the border of the neural plate and is dependent on signaling molecules from the surrounding neuroepithelium and underlying mesoderm (LaBonne and Bronner-Fraser, 1998, 1999). Bone morphogenetic proteins (BMPs), Wnts, fibroblast growth factors (FGFs) and retinoic acid (RA) are key signaling molecules of the aforementioned morphogenetic processes. Specifically, BMP specifies the NCC site in the lateral border of the neural plate, while concerted actions of Wnts, FGFs and RA convert the specified cells into NCCs (Barembaum and Bronner-Fraser, 2005; Steventon et al., 2005). EMT in NCCs production is modulated by a number of transcription factor families that includes Snail, Sox, and endothelins (Ets) gene families, which regulate cell–cell and cell–matrix adhesion and the detachment of NCCs from the

neuroepithelium (Theveneau and Mayor, 2012).

Cranial NCCs can contribute to the craniofacial skeleton, cranial ganglia of the sensory nervous system, enteric nervous system, Schwann cells, wall of the aorta and the cardiac septa (Cordero et al., 2011). Consequently, abnormal development of the neural crest would lead to congenital malformations in the fetus that include neural tube defects (NTD), atrioventricular septal defects, persistent ductus arteriosus, and Waardenburg syndrome (Weston, 1981). In this study, we investigated the effects of ethanol exposure on cranial NCC development and bioactivities. Early chick embryos (before HH10) were used as an experimental model and also 2% ethanol (effective ethanol dosage determined in our reported study (Li et al., 2015)).

2. Materials and Methods

2.1 Chick embryos

Fertilized leghorn eggs were obtained from the Avian Farm in South China Agriculture University (Guangzhou, China). All chick embryos were staged according to the Hamburger-Hamilton (HH) scheme (Hamburger and Hamilton, 1951). Ethanol (Guangzhou chemical reagent factory, China) was introduced into the embryos by two routes. For ethanol treatment of early embryos, HH1 chick embryos were incubated in EC culture (Etchevers, 2011; Streit, 2008) in the presence of 0.719% simple saline (control) or 2% ethanol inside a humidified incubator (Yiheng Instrument, Shanghai, China) set at 38°C and 70% humidity. The embryos were extracted from these incubated eggs for analysis when the embryos reached the desired developmental (HH) stages. For alizarin red staining of whole embryos, eggs that have been incubated for 3 days were windowed and injected with either 2% ethanol or equivalent volume of saline into the albumen. The injections were given once every 1.5 days, sealed with sellotape and then incubated for a further 11 days. The embryos were harvested when they reached the desired stage, according to experimental requirements.

2.2 Primary cranial NCCs cultures

NCCs were prepared from the cranial neural tube explants isolated from chick embryos, according to methods previously described (Etchevers, 2011). Briefly, fertilized chick eggs were incubated until the embryo reached 10 somite stage (HH10). The cranial neural tubes were then dissected from the head region of the embryo and explanted into 3.5mm dishes. The explants were cultured in DMEM and 10% FBS for 6 hours at 37ºC and 5% CO_2, to allow the explants to adhere. The explants were then incubated until some NCCs were observed migrating out of the neural tubes. At this stage, 1ml of culture medium containing 0.719% simple saline (control) or 2% ethanol were introduced into the cultures. These saline and ethanol treated explants were allowed to develop for 48 hours and then the areas containing the migrant NCCs were measured and analyzed using Image-Pro Plus 7.0 software. After incubation, the NCCs cultures were washed with pre-warmed PBS and fixed in 4% paraformaldehyde (PFA) overnight at 4°C.

2.3 Alizarin red staining of whole embryos

The craniofacial skeleton was visualized in 14-day (E14) chick embryos by staining with alizarin red dye (Solarbio, Beijing, China). Briefly, the embryos were fixed in 95% ethanol for 3 days, then the skin and viscera were carefully removed before they were post-fixed in 95% ethanol for one day. The embryos were then treated with 0.5% KOH (Jinan University, Guangzhou, China) for 48 hours and stained in 0.001% alizarin red dye for 3 days. Finally, the embryos were cleared in a graded series of glycerol (diluted with water) and the craniofacial skeleton photographed using a stereomicroscope (Olympus MVX10, Japan).

2.4 Bromodeoxyuridine incorporation assay

To determine the extent of cell proliferation, 0.03mg/ml of Bromodeoxyuridine (BrdU, Roche diagnostics, USA) was introduced into the saline- and ethanol-treated chick embryos for 2 hr. The embryos were then harvested, fixed in 4% paraformaldehyde, and then stained with a BrdU-specific monoclonal antibody

(1:100,BD Biosciences, USA), according to the manufacturer's instruction (Roche Diagnostics Corporation). For the quantification in the BrdU experiments, we manually counted $BrdU^+$ and $HNK1^+$ cells at the neural tube.

2.5 Immunofluorescent staining

Chick embryos were harvested form fertilized eggs after incubation and fixed in 4% PFA (Guangzhou chemical reagent factory, China) overnight at 4ºC. Immunofluorescent staining were performed on whole-mount embryos and primary explants using the following antibodies: AP-2α (1:250 dilution, DSHB, USA), Pax7 (1:200, DSHB, USA), HNK-1 (1:400, Sigma, USA), pHIS3 (1:400, Santa Cruz, USA), c-Caspase3 (1:200, Cell Signaling Technology, USA), Laminin (1:200, Sigma, USA), N-Cadherin (1:200, DSHB, USA), Cadherin 6B (1:100, DSHB, USA), and Cadherin 7 (1:100, DSHB, USA). Briefly, the fixed chick embryos were incubated with the primary antibodies at 4ºC overnight on a shaker. After extensive rinsing in PBST (0.1% Tween-20), the embryos were treated with a corresponding Alexa Fluor® 488 or 555 labelled secondary antibody (1:1000, Invitrogen, USA) at 4ºC overnight on a shaker. For double immunofluorescent staining, the primary antibodies were incubated one after the other and then the two corresponding secondary antibodies were mixed and incubated together. All the embryos were later counterstained with DAPI (1:1000, Invitrogen) at room temperature for 1 hour. Subsequently, the stained embryos were embedded in gelatin and sucrose (7.5% gelatin in 15% sucrose-PBS) and frozen at -80ºC, thensectioned at 10μm using a cryostat (Leica CM1900).

2.6 In situ hybridization

Whole-mount *in situ* hybridization was performed on chick embryos according to previously described standard protocol (Henrique et al., 1995). The RNA antisense Cadherin 6B, Msx1, FOXD3 and BMP4 probes used were obtained by the reverse transcription - polymerase chain reaction (RT-PCR) technique as described by Bales et al. (1993) (Bales et al., 1993). Total RNA was isolated from 72-h chicken embryos. Digoxigenin-labeled antisense probes were synthesized to specifically detect Cadherin

6B, Msx1, FOXD3 and BMP4 mRNAs. The primers used to generate the in situ hybridization probes are shown in supplementary Figure S1. Briefly, digoxigenin-labelled probe was synthesized for Slug (Nieto et al., 1994). The whole-mount stained embryos were photographed and then frozen sections, at thickness of 20μm, were prepared from these embryos for histological examination.

2.7 RNA isolation and RT-qPCR analysis

Total RNA was isolated from the heads of HH10 chick embryos (N>25 embryos） using a Trizol kit (Invitrogen, USA) and protocol according to the manufacturer's instructions. First-strand cDNA synthesis and SYBR® Green qPCR assay were performed using a PrimeScript™ RT reagent kit (Takara, Japan). All specific primers of our genes of interest used in this study are listed in Supplementary Figure S2. Reverse transcription and amplification reactions were performed inside Bio-Rad S1000™ (Bio-Rad, USA) and ABI 7000 thermal cyclers. Housekeeping gene GAPDH was run in parallel with the genes of interest to confirm that equal amounts of RNA has been used in each reaction. The relative expression level of our gene of interest was determined after normalization with GAPDH. The RT-qPCR results were determined from three independent sets of experiments.

2.8 Photography

Following immunofluorescent staining, whole mount embryos and regions of interest were photographed using a stereo-fluorescence microscope setup and processed using an Olympus software package Image-Pro Plus 7.0. The embryos were then sectioned at 10μm on a cryostat microtome (Leica CM1900), photographed using an Olympus IX51epi-fluorescent microscope (at 200x and 400x) and analyzed using a CW4000 FISH Olympus software.

2.9 Data analysis

All data analyses and graphics were performed using the Graphpad Prism 5 software (Graphpad Software, CA, USA). The results were presented as the mean

value (**{EMBED Equation.3}**± SE). All data were analyzed using the t-test to establish difference between the experimental and control groups. $P<0.05$ was considered to be significantly different.

3. Results

3.1 Ethanol exposure induces craniofacial dysplasia in chick embryos

HH20 (three-day-old) chick embryos were exposed to 2% ethanol (once every 1.5 days) or equivalent volume simple saline into the albumen and harvested for analysis on the 14^{th} day of incubation (Fig 1A). It was determined that exposing early chick embryos to ethanol (Fig. 1C) significant increased the incidence of parietal defects and premaxilla shortened as compared with saline-treated (control) embryos (Fig. 1B). The skulls of these 14-day-old embryos were also stained with alizarin red dye to establish whether ethanol exposure affected craniofacial osteogenesis. The results revealed that there were distinct pattern of developmental bone defects, especially on the parietal bone (PA) (Figs. 1B1-C1, 1D) and premaxilla length (control: 3.57±0.20mm, n=6; Ethanol: 2.63±0.32mm, n=8; $p<0.05$; Figs. 1B2-C2, 1E). of embryos treated with 2% ethanol. The ethanol exposure also increased embryo mortality (Fig. 1F), reduced embryos' body weight (control: 2.46±0.29g, n=10; Ethanol: 1.54±0.20g, n=17; $p<0.05$; Fig. 1G).

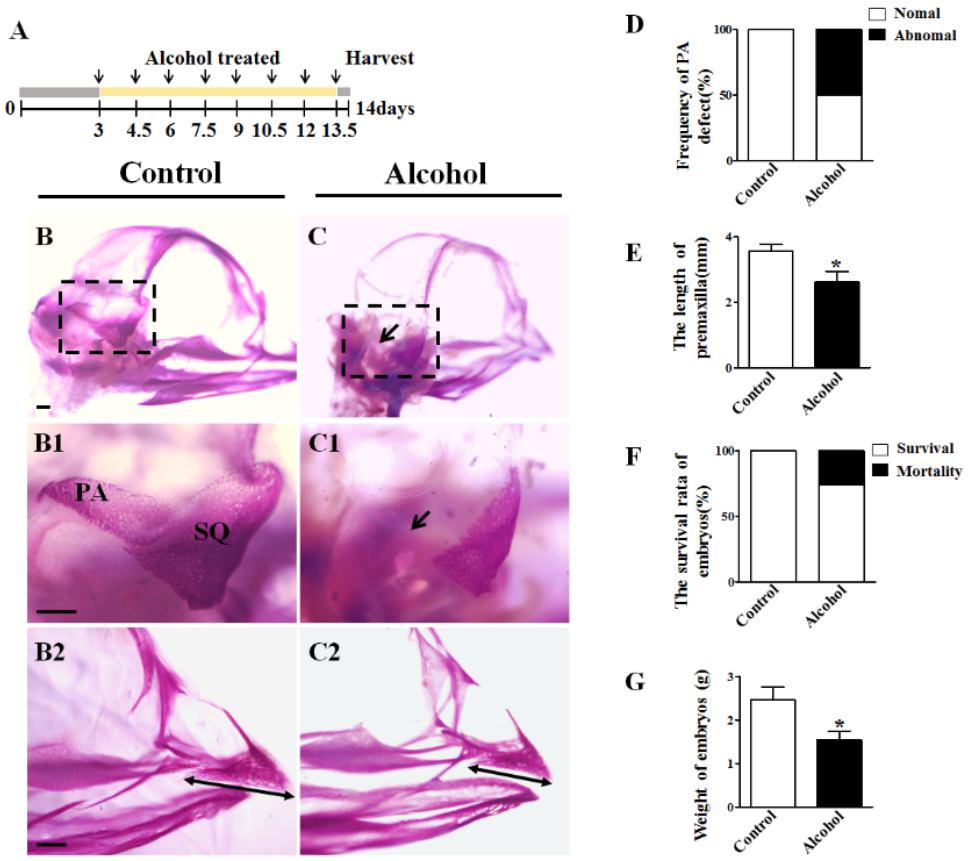

Fig 1. Ethanol exposure disrupts craniofacial ossification in chick embryos.

A: Schematic diagram showing 3-day pre-incubated chick embryos injected with either 2% ethanol or simple saline (control) *in vivo*, once every 1.5 days for 11 days. **B-C**: Representative appearance of the skulls of saline- and ethanol-treated embryos was stained with Alizarin Red S, respectively. **B1−C1, B2-C2**: showing higher magnification images of regions highlighted by the dotted outlined squares and premaxilla in B-C, respectively. Bar charts incidence of parietal bone defects (**D**), comparing the length of premaxilla (E), survival rate (**F**) and comparing the weights (**G**) between control and ethanol-treated embryos. Abbreviations: PA, parietal bone; SQ, squamosal bone. Arrow (C, C1): PA. Scale bars = 1 mm in B-B2 and C-C2.

3.2 Ethanol exposure inhibits cranial neural crest formation during early chick embryo development

The craniofacial bones are derived from cranial NCCs during neurulation (Santagati and Rijli, 2003). Hence, we examined whether defects in craniofacial

skeleton following exposure to ethanol were attributed to abbreviated cranial NCCs generation and differentiation. Early chick embryos were exposed to 2% ethanol or simple saline (control) during EC culture for 36 hours (Fig. 2A). Ap-2α and Pax7 antibodies were used as markers for cranial NCCs (Figs. 2B-D) and pre-migratory/migratory NCCs (Figs. 2E-G), respectively. The immunofluorescent staining revealed that the number of Ap-2α^{+} cranial NCCs were significantly reduced by the ethanol exposure, as seen in whole-mount preparations (Figs. 2B-D, H; control $41.58\pm1.70\times10^{3}\mu m^{2}$, n=23; alcohol $33.96\pm1.99\times10^{3}\mu m^{2}$, n=23; $p<0.01$) and transverse tissue sections (Figs. 2B1-D1 and B2-D2). Similarly, the number of Pax7^{+} NCCs were also significantly decreased following ethanol treatment as evident in whole-mount preparations (Figs. 2E-G, I; con: $45.80\pm3.39\times10^{3}\mu m^{2}$, n=9; alcohol: $32.26\pm3.92\times10^{3}\mu m^{2}$, n=8; $p<0.05$) and transverse tissue sections (Figs, 2E1-G1, E2-G2). These results indicate that alcohol exposure directly affect the cranial neural tube and NCCs (Fig. 2J).

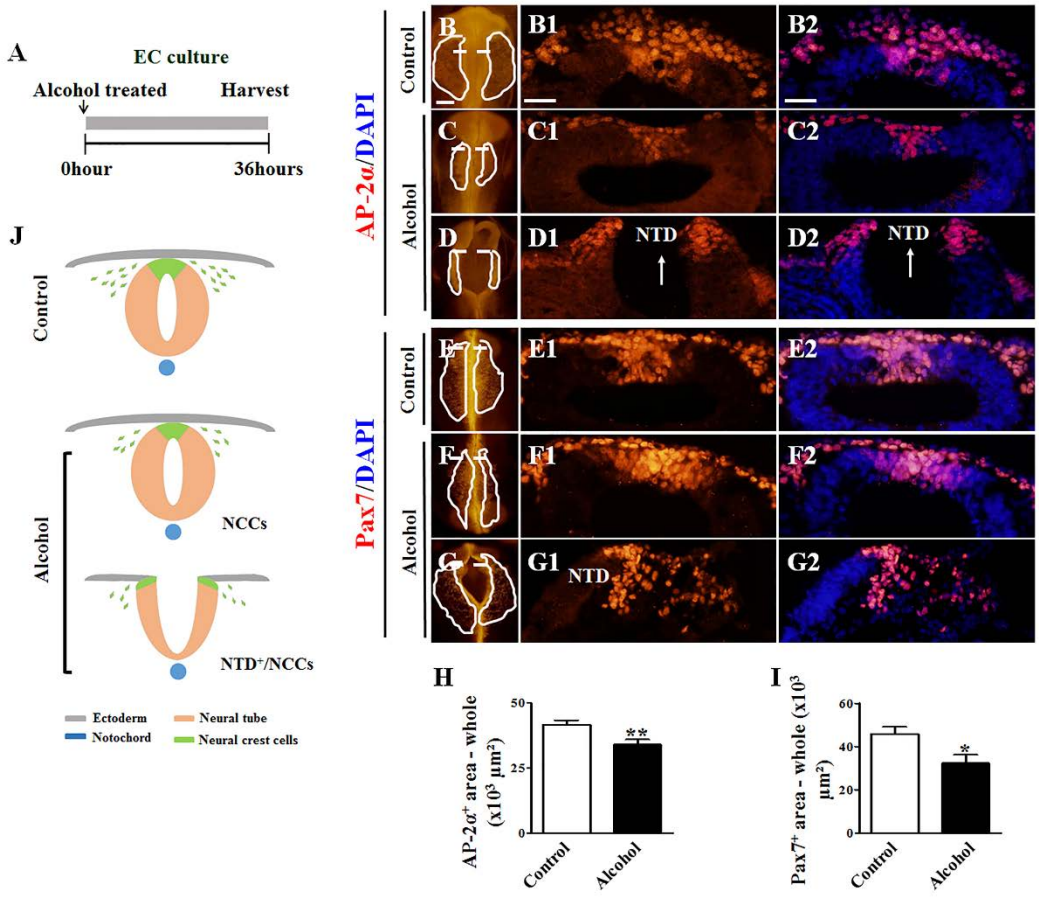

Fig 2. Ethanol reduces cranial neural crest delamination in chick embryos

A: Schematic diagram showing chick embryo exposure to saline (control) and 2% ethanol for 36 hours in EC culture. The cultured embryos were immunofluorescently stained for AP-2α and Pax7 expression. **B-D**: Representative images of the cranial regions of control and ethanol-treated embryos immunofluorescently stained with AP-2α (**B-D**) and Pax7 (**E-G**) antibodies. **B1-G1**: Transverse sections were produced from the embryos in B-G (as indicated by the dotted lines). **B2-G2**: The transverse sections of **B1-G1** were counterstained with DAPI, respectively. Bar charts comparing the AP-2α^{+} (**H**) and Pax7^{+} (**I**) areas of control and ethanol-treated embryos. **J**: Schematic diagram showing the impact of saline and ethanol exposure on cranial NCC production and morphology of the neural tube. Scale bars = 200 μm in B-G and 50 μm in B1-G1 and B2-G2.

Immunofluorescent staining was also performed to reveal HNK-1 (a marker for migratory NCCs) expression. The staining show tissue areas containing HNK-1^{+} migratory NCCs were significantly reduced in presence of 2% ethanol as compared with the control (Figs. 3A-D; con: $44.41\pm1.08\times10^{3}\mu m^{2}$, n=12; alcohol: $34.13\pm3.42\times10^{3}\mu m^{2}$, n=12; $p<0.05$) and transverse sections (Figs. 3A1-C1, A2-C2, D; control: $32.03\pm1.32\times10^{4}\mu m^{2}$, n=30; alcohol: $18.95\pm2.42\times10^{4}\mu m^{2}$, n=30, $p<0.001$). The result suggests that ethanol can disrupt NCCs migration. In support, we employed an *in vitro* neural tube explant model where NCCs migration from the neural tube was assessed in presence and absence of ethanol (Fig. 3E). Compared with the control, the area containing migratory NCCs was significantly smaller (Figs. 3F-H; control: $416.2\pm41.71\times10^{4}\mu m^{2}$, n=24; alcohol: $225.1\pm36.34\times10^{4}\,\mu m^{2}$, n=22, $p<0.01$). Moreover, the numbers of HNK-1^{+} NCCs from the neural tube explants were also reduced in presence of ethanol (Figs. 3F1-G1, I; control: 0.785±0.022, n=30; alcohol: 0.455±0.037, n=30, $p<0.001$), which further validate the Ap-2α and Pax7 observations.

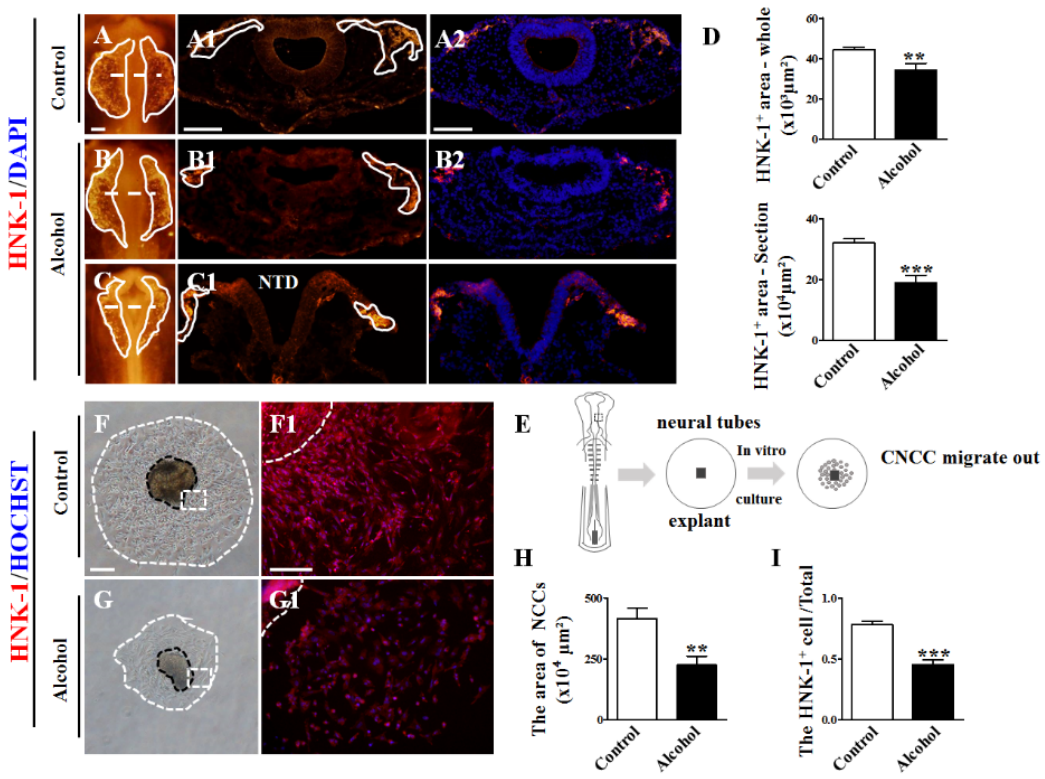

Fig 3. Ethanol reduces cranial neural crest cell migration in chick embryos

A-C: Representative micrographs of the cranial regions of control and ethanol-treated chick embryos immunofluorescently with HNK-1 antibodies. **A1-C1**: Transverse sections produced from A-C, as indicated by the dotted lines, and counterstained with DAPI dye (**A2-C2). D**: Bar charts comparing the size of HNK-1^{+} areas in whole-mount (A-C, the upper chart) and histological sections (A1-C1, the lower chart) of control and ethanol-treated embryos. **E**: Schematic diagram showing how cranial neural tube explants were isolated from HH10 chick embryos, and then exposed *in vitro* to saline (control) or 2% ethanol for 48 hours. **F-G**: Representative bright-field images of control and ethanol-treated neural tube explants after 48-hours culture. **F1-F2**: control and ethanol-treated neural tube explants, immunofluorescently stained for HNK-1 after 48-hours culture. Bar charts comparing the areas occupied by migratory neural crest cells (**H**) and the ratios of HNK-1^{+} cells over total cell number (**I**) between control and ethanol-treated cultures. Abbreviation: CNCCs, cranial neural crest cells. Scale bars = 200 μm in A-C; 100 μm in A1-C1, A2-C2; 400 μm in F-G and 100 μm in F1-G1.

3.3 Effects of ethanol exposure on cranial NCC proliferation and apoptosis

We investigated whether ethanol affected NCC survival. Double immunofluorescent staining was performed on control and ethanol-treated embryo sections for Ap-2α and pHIS3 expression. The results revealed a significant reduction in the number of proliferating cranial NCC (co-expressing Ap-2α and pHIS3) between ethanol-treated and non-treated neural tubes (control: 4.00±0.35, n=17; ethanol: 2.35±0.39, n=17; $p<0.01$; Figs. 4A-D). Moreover, we have used BrdU incorporation assay to further validate that ethanol was able of repressing cranial NCC proliferation. The results showed that there were significantly fewer $BrdU^{+}$ cranial NCCs in the ethanol-treated embryos than the control (control: 13.83±1.07, n=12; ethanol: 9.67±1.20, n=12; $p<0.05$; Figs, 4E-H).

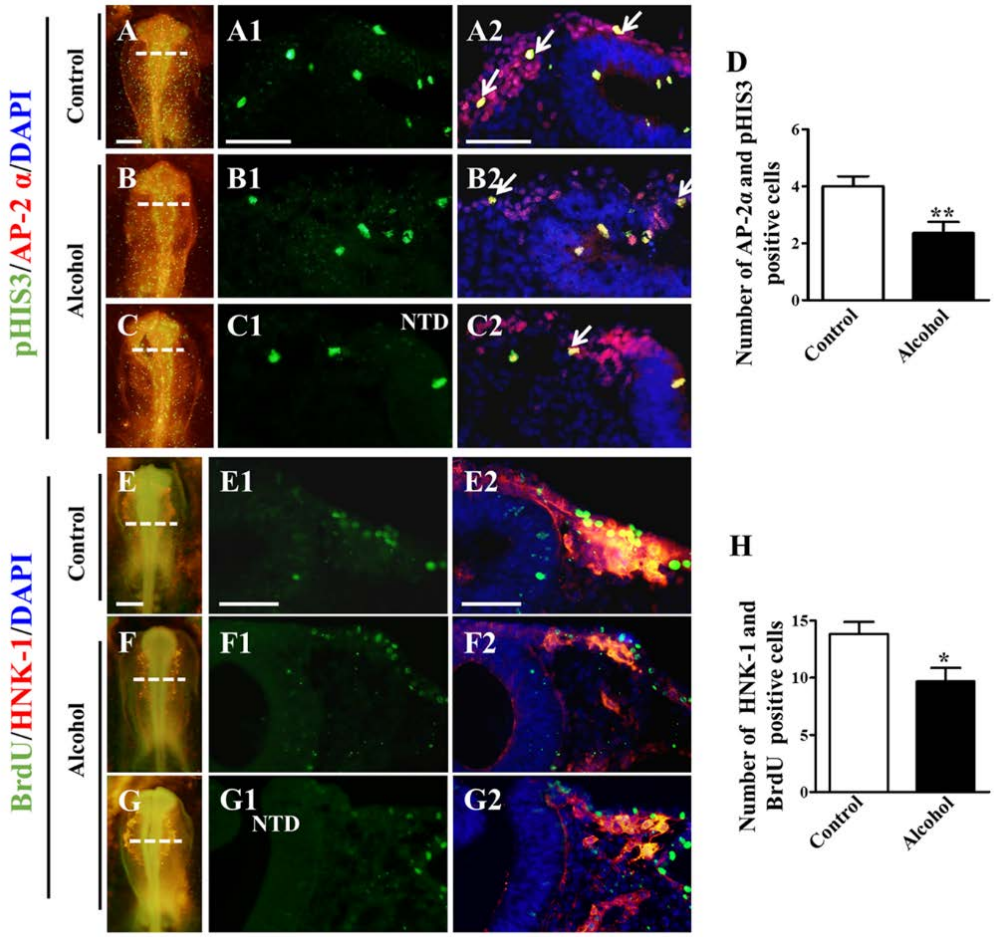

Fig 4. Ethanol exposure inhibits cranial neural crest cell proliferation in chick embryos

A-C: Representative micrographs of the cranial regions double immunofluorescently with pHIS3 (green) cell proliferation marker and AP-2α (red) neural crest cell marker in control (A) and ethanol-treated (B-C) embryos. **A1-C1**: transverse sections produced from A-C at the level indicated by the dotted white lines. **A2-C2**: are sections of A1-C1 counterstained with DAPI dye, respectively. **D**: Bar chart comparing the number of $pHIS3^{+}$ /$AP\text{-}2\alpha^{+}$ cell between control and ethanol-treated embryos. **E-G**: Representative micrographs showing extent of BrdU incorporation (green) in control and ethanol-treated early chick embryos. **E1-G1**: are transverse sections were generated from E-G respectively. **E2-G2**: sections of E1-G1 were double immunofluorescently stained of HNK-1 (red) neural crest cell marker and BrdU. All sections were counterstained with DAPI. **H**: Bar chart comparing the number of $BrdU^{+}$/ $HNK\text{-}1^{+}$ in control and ethanol-treated embryos. Scale bars = 200 µm in A-G; 100 µm in A1-G1 and A2-G2.

Double immunofluorescent staining was performed for c-Caspase3 and Ap-2α to establish the extent of cranial NCCs cell death following ethanol treatments. It was determined that there were significantly more NCCs co-expressing Ap-2α and c-Caspase3 in ethanol-treated than saline-treated embryos (control: 4.43±0.66, n=14; ethanol: 7.31±0.63, n=13; $p<0.01$; Figs. 5A-D). Furthermore, *in vitro* neural tube explant studies (as described in Fig. 3E) also showed that the numbers propidium iodide labeled neural crest cells (migrated out from neural tube explants) significantly increased following ethanol treatment (control: 0.37±0.07, n=12; alcohol: 0.79±0.0423, n=12; $p<0.001$; Figs. 5E-G). These results suggest that ethanol treatment inhibited and increased cranial NC cell proliferation and apoptosis, respectively.

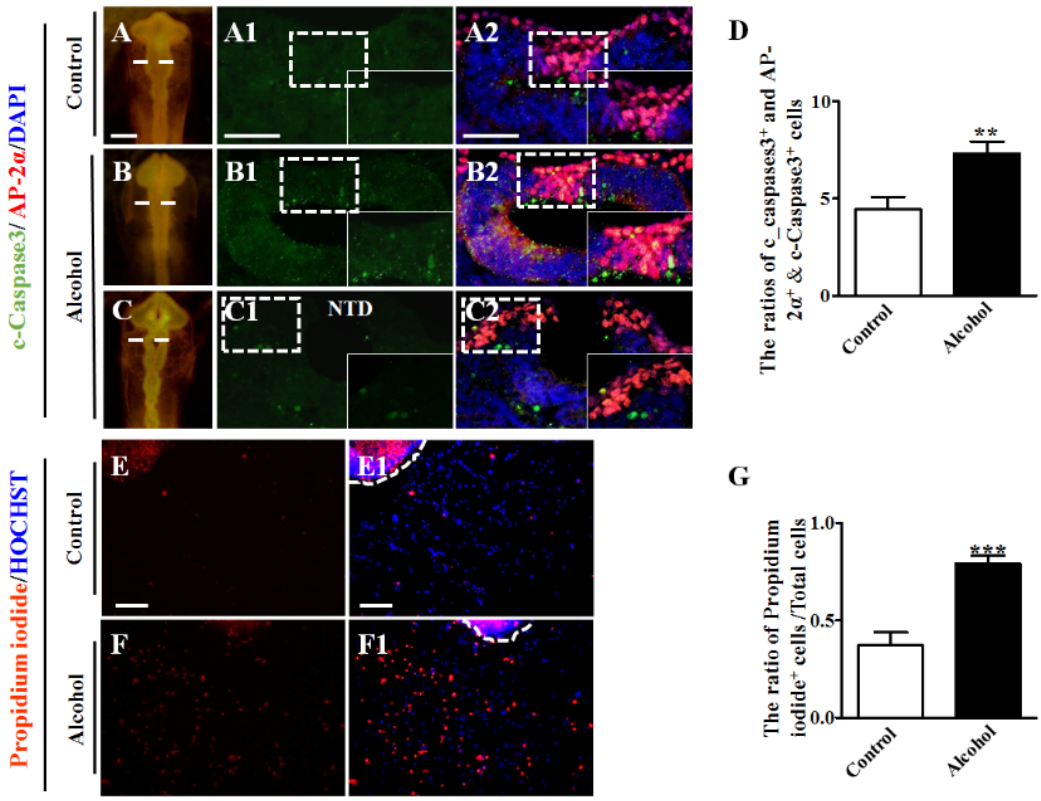

Fig 5. Ethanol exposure increases apoptosis in cranial neural crest cells

A-C: Representative micrographs of the cranial regions double immunofluorescently for c-Caspase3 (green, apoptotic marker) and AP-2α (red, NCC marker) in control (A) and ethanol-treated (B-C) embryos. **A1-C2**: transverse sections of A-C (dotted lines) showing the presence of c-Caspase3^{+} and AP-2α^{+} in control and ethanol-treated embryos. **E-F1**: Representative micrographs of the cranial region (isolated from HH10 chick embryos) stained with propidium iodide and Hochst dyes. Bar charts comparing the number of c-Caspase3^{+}/AP-2α^{+} cells (**D**) and propidium iodide/total cells (**G**) in control and ethanol-treated embryos. Scale bars = 200 µm in A-C; 100 µm in A1-C1 and A2-C2; 200 µm in E-F and E1-F1.

3.4 Ethanol exposure represses the EMT process during NCC delamination

Initiation of EMT at the dorsal side of the neural tube is indispensable step for NCC delamination. EMT is accompanied with a down-regulation of adhesion molecules and extra cellular matrix expression, for example N-Cadherin and Laminin (Padmanabhan and Taneyhill, 2015; Scarpa et al., 2015). We examined the expression

of laminin in the dorsal neural tube of ethanol-treated embryos. It was determined that laminin did not down-regulated in the neural tube compared with the control, as indicated by arrows (Figs. 6A-C, A1-C1, A2-C2). In addition, immunofluorescent staining for N-Cadherin revealed that it was more strongly expressed in the dorsal neural tubes of ethanol-treated embryos than control embryos, as indicated by arrows (Figs, 6D-F, D1-F1, D2-F2). RT-qPCR analysis was performed to investigate how ethanol affected the N-Cadherin and E-Cadherin transcription. The results indicated that ethanol exposure increased N-Cadherin and E-Cadherin expressions expression (N-Cadherin: control: 1.00±0.23, n=3; alcohol: 2.22±0.23, n=3; $p<0.05$; E-Cadherin: control: 1.00±0.18, n=3; alcohol: 2.45±0.33, n=3; $p<0.05$; Fig. 6G).

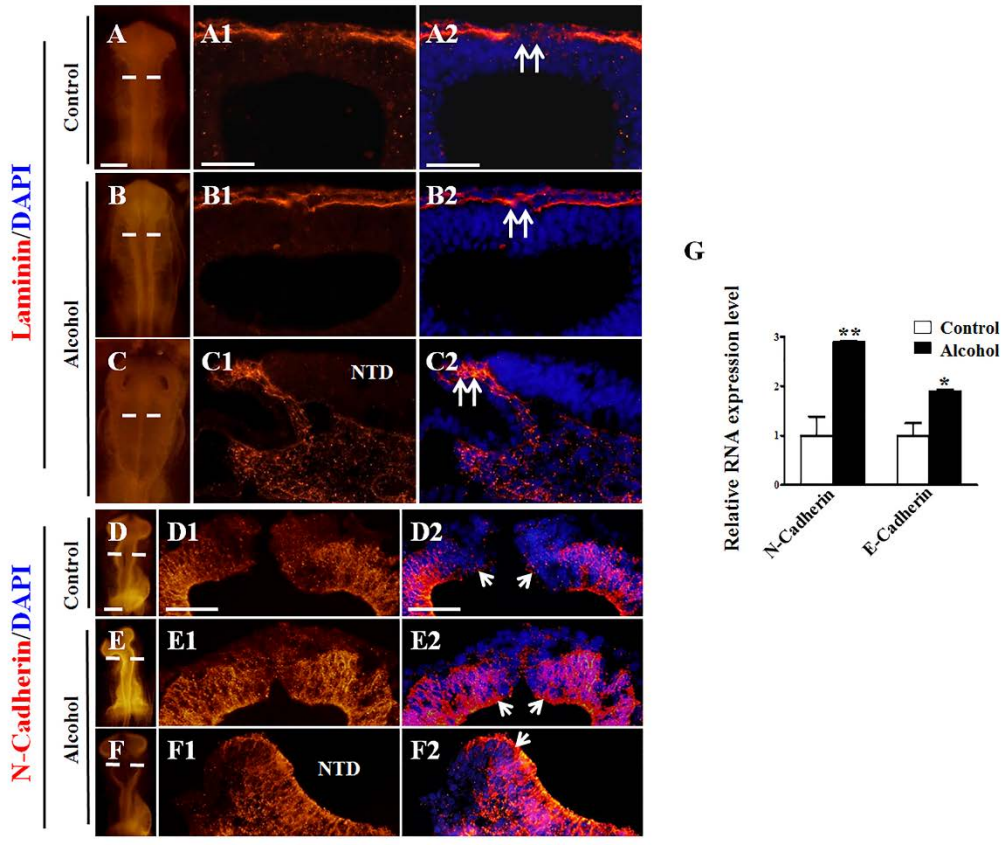

Fig 6. Effects of ethanol exposure on Laminin and N-Cadherin expression

A-C: Representative micrographs of the cranial regions of control and ethanol-treated chick embryos immunofluorescently stained for Laminin. **A1-C1**: Transverse sections produced from A-C, as indicated by the dotted lines and counterstained with DAPI dye (**A2-C2**). **D-F**: Cranial regions of chick embryos immunofluorescently for N-Cadherin. **D1-F1**: Transverse sections of D-F, as indicated

by the dotted lines, counterstained with DAPI dye (**D2-F2**). **G**: RT-qPCR analysis showing the extent of Laminin, N-Cadherin and E-Cadherin expression following ethanol treatment. Expression was normalized against GAPDH. Scale bars = 200 μm in A-C, D-F and 50 μm in A1-C1, A2-C2, D1-F1, D2-F2.

In situ hybridization was used to show Cadherin 6B expression. The results demonstrated that Cadherin 6B expression was dramatically up-regulated following ethanol treatment (Figs. 7B-B2) as compared with saline treatment (Figs. 7A-A2). Moreover, we immunofluorescently stained the embryos with Cadherin 6B and Cadherin 7 antibodies (Chalpe et al., 2010) to show the expression of these proteins in the tissues (Figs. 7C-H). The results showed that Cadherin 6B was more strongly expressed in dorsal neural tube of ethanol-treated embryos than control embryos (Figs. 7C-E, C1-E1, C2-E2, I) (control: $6.51\pm1.02\times10^4$ μm^2, n=16; alcohol: $9.90\pm0.82\times10^4$ μm^2, n=16; $p<0.05$). For Cadherin 7 (trans-adhesion molecule of Cadherin 6B) expression, it was significantly down-regulated in the neural tubes of ethanol embryos compared with control embryos (Figs, 7F-H, F1-H1, F2-H2, J) (control: $8.08\pm0.43\times10^4\mu m^2$, n=18; alcohol: $6.00\pm0.29\times10^4\mu m^2$, n=18; $p<0.001$). These results suggest that abnormal expression of adhesion molecules might be partly responsible for the observed NCCs dysplasia induced by ethanol exposure. RT-qPCR analysis was performed to validate ethanol affected Cadherin 6B and Cadherin 7 transcription (Cadherin 6B: control: 1.00±0.04, n=3; alcohol: 3.35±0.45, n=3; $p<0.01$; Cadherin 7: con: 1.00±0.10, n=3; alcohol: 0.33±0.06, n=3; $p<0.01$; Fig. 7K).

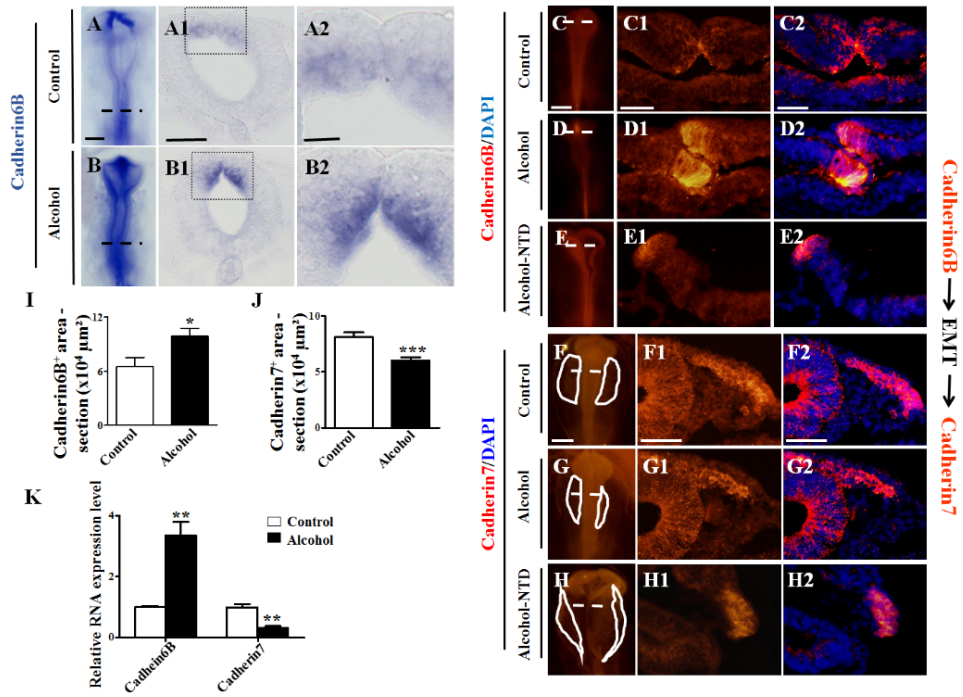

Fig 7. Effects of ethanol exposure on Cadherin 6B and Cadherin7 in neural tubes

A- B: *In situ* hybridization showing Cadherin 6B expression at the cranial neural tubes of control and ethanol-treated chick embryos. **A1-B1**: Transverse sections produced from A-B, as indicated by the dotted lines. **A2-B2**: Higher magnification of regions indicated by dotted squares in A1-B1, respectively. **C-E**: Representative images of the cranial regions of control and ethanol-treated chick embryos immunofluorescently stained for Cadherin 6B expression. **C1-E1**: Transverse sections produced from C-E, as indicated by the dotted lines, and counterstained with DAPI dye (**C2-E2). F-H**: Cranial regions of control and ethanol-treated chick embryos immunofluorescently stained for Cadherin 7 expression. **F1-H1**: Transverse sections produced from F-H, as indicated by the dotted lines and counterstained with DAPI dye (**F2-H2**). **I-J**: Bar charts comparing the size of Cadherin $6B^+$ areas (I) or Cadherin 7^+ areas (J) in transverse sections of control and ethanol-treated embryos. **K**: RT-qPCR analysis showing the extent of Cadherin 6B and Cadherin 7 expression. Expression was normalized against GAPDH. Scale bars = 200 µm in A-B, C-E, F-H; 50 µm in A1-B1; 20 µm in A2-B2 and 50 µm in C1-E1, C2-E2, F1-H1, F2-H2.

Msx1 and BMP4 are important regulators of key adhesion molecules during EMT (Jia et al., 2016). Hence, *in situ* hybridization and RT-qPCR analysis for *Msx1* and *BMP4* were conducted on ethanol-treated and non-treated embryos. The results revealed ethanol exposure significantly inhibited both Msx1 and BMP4 expression in the dorsal neural tubes (Fig. 8) (BMP4: control: 1.00±0.06, n=3; alcohol: 0.41±0.04, n=3; $p<0.01$; MSX1: control: 1.00±0.10, n=3; alcohol: 0.39±0.05, n=3; $p<0.01$; Fig. 8G).

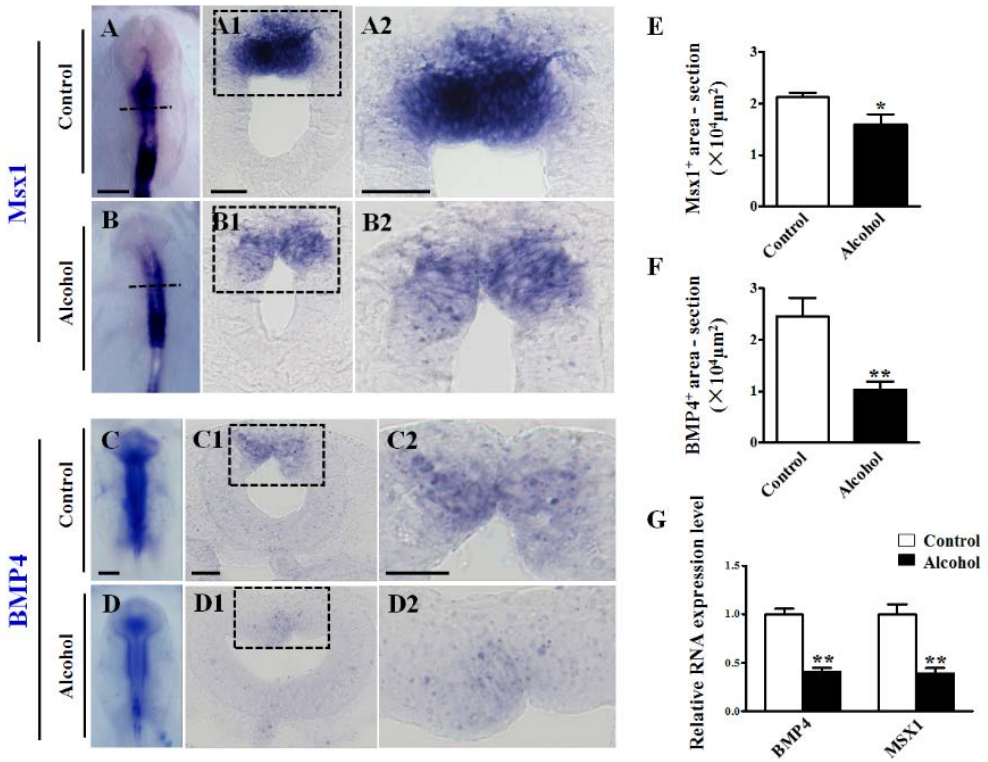

Fig 8. Effects of ethanol exposure on Msx1 and BMP4 expression in neural tubes

A-D: *In situ* hybridization showing the extent of Msx1 and BMP4 expression in the cranial neural tube of control and ethanol-treated embryos. **A1-D1**: Transverse sections of the stained embryos at the levels indicated by the dotted lines in **A2-D2**: Higher magnification of regions indicated by dotted squares in A1-D1, respectively. **E-F**: Bar charts comparing the size of $Msx1^+$ (E) and $BMP4^+$ (F) areas in transverse sections of control and ethanol-treated embryos. **G**: RT-qPCR analysis showing the extent of Msx1 and BMP4 expression. Expression was normalized against GAPDH. Scale bars = 200 μm in A-D; 50μm in A1-D1 and 50 μm in A2-D2.

In situ hybridization and RT-qPCR analysis were also performed for Slug and FoxD3 expression. It was found that ethanol treatment significantly down-regulated the expression of both genes in the dorsal neuroepithelium (Slug: control: 1.00±0.04, n=3; alcohol: 0.28±0.03, n=3; $p<0.001$; FoxD3: con: 1.00±0.05, n=3; alcohol: 0.38±0.36, n=3; $p<0.01$; Fig. 9G). The results suggest that ethanol exposure impedes EMT by de-regulating the expression of up-stream transcription factors (Thiery and Sleeman, 2006).

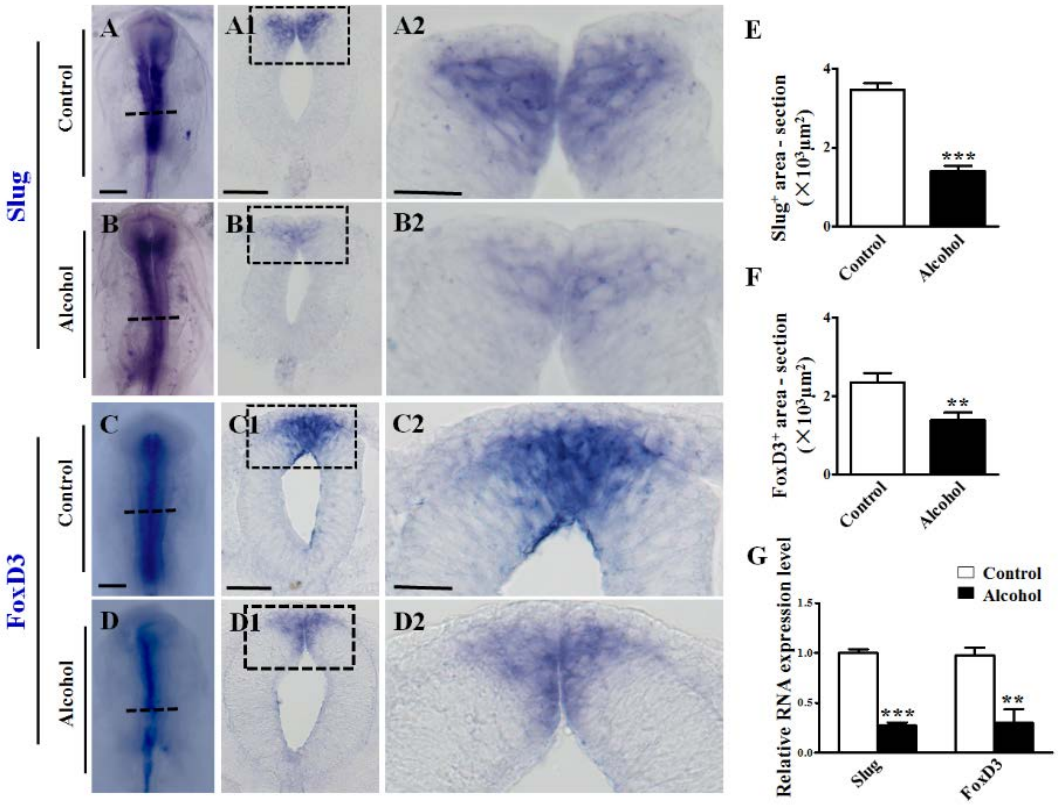

Fig 9. Effects of ethanol exposure on Slug and FoxD3 expression in the neural tube

A-B: Representative micrographs of Slug and FoxD3 expression in the cranial regions of control and ethanol-treated embryos. **A1−D1**: Transverse sections of the stained embryos at the levels indicated by the dotted lines in A−D. **A2-D2**: Higher magnification of regions indicated by dotted squares in A1-D1, respectively. **E-F**: Bar charts comparing the size of $Slug^+$ (E) and $FoxD3^+$ (F) areas in transverse sections of control and ethanol-treated embryos. **G**: RT-qPCR analysis showing the extent of Slug and FoxD3 expression. Expression was normalized against GAPDH. Scale bars = 200

μm in A-D; 50μm in A1-D1 and 25μm in A2-D2.

4. Discussion

Consumption of alcohol during pregnancy is now recognize as a potential teratogenic risk and FAS is the most severe developmental defects that could result (Gupta et al., 2016). Craniofacial malformations which can be caused by excess alcohol consumption during pregnancy, actually comprise of one-third of all congenital birth defects (Dixon et al., 2006). Presently, we investigated the underlying pathogenic mechanism of craniofacial abnormalities - using early chick embryos exposed to 2% ethanol as a model. The avian embryo is a good model to use because the ethanol can directly affect the embryo instead of having first to pass through the mammalian placenta and distribute in amniotic cavity (Brien et al., 1985; Tranmer, 1985). In this study, the first observable abnormal phenotype, induced by ethanol exposure, is an increased incidence of parietal bone defects and suppressed the growth of rostrum in the chick embryos (Figure 1). The craniofacial skeleton undergoes osteogenesis by intramembranous ossification, where the bone is directly formed from mesenchymal stem cells predominantly derived from cranial NCCs (Santagati and Rijli, 2003). In this context, we focused on the cranial NCCs to understand how ethanol affects craniofacial development in the chick embryo.

It was determined that ethanol exposure induced two kinds of phenotypes in the early chick embryo. Firstly, there was a reduction in cranial NCCs production without NTD (mild malformation) and secondly NTD accompany with a reduction in cranial NCCs production (severely malformation). The ethanol exposure induced NTD phenotype in chick embryo is consistent with perilously literatures (Giles et al., 2008). We speculate that the failure of the neural tube closure was attributed to extensive apoptosis following ethanol exposure. Ethanol exposure suppressed the cranial NCCs production is a certain degree of specificity since we still could observe it even in the mild malformation group. Therefore, we focused all attention on the cranial NCCs using AP-2α (expressing cranial NCC become neural or mesenchymal NCC derivatives) (Minarcik and Golden, 2003) and Pax7 (specific for cranial NNCs

pre-migratory and migratory) (Monsoro-Burq, 2015) antibodies (Figure 2). Our finding is consistent with previously reported observations but the authors did not showed the type of NCCs affected by the ethanol because the antibodies were not available(Cartwright and Smith, 1995).

The difference between control and ethanol-treated embryos could also be attributed to ethanol affecting cranial NCC survival during development. Hence, we also evaluated the effects of ethanol on cranial NCCs proliferation and apoptosis using double immunofluorescent staining - for proliferation pHIS3/AP-2α antibodies (Figure 4) and for apoptosis c-Caspase3/AP-2α antibodies (Figure 5). Ahlgren et al. (2002) described the analysis of HNK-1 and of apoptosis markers following ethanol exposure (Ahlgren et al., 2002) and our results confirmed their study (Figure 3). The results imply that alterations in these 2 morphogenetic processes could account for how ethanol reduces the number of cranial NCCs in the embryos. Cartwright et al. have reported that ethanol could activate the endogenous cell death pathway in NCCs (Erickson et al., 1989). Further precise experiments are certainly required to address the NCCs death mechanism induced by ethanol.

Besides apoptosis, Oyedele et al. reported that the actin cytoskeleton of avian cranial NCCs and their ability to migrate were negatively affected by exposure to ethanol *in vitro* culture (Oyedele and Kramer, 2013). Likewise, we demonstrated that ethanol treatment reduced the number of HNK-1^+ (migratory (Erickson et al., 1989)) cranial NCCs in chick embryos and *in vitro* neural tube explant model (Figure 3). This suggests that the inhibitory effect of ethanol on cranial NCC production is also manifested in NCC induction, delamination, and migration. This also implies that the ethanol-induced parietal and squamosal bone defects could be partly attributed to a deficit in cranial NCC production.

EMT and adherens junction disassembly by pre-migratory NCCs are prerequisite processes for NCC generation (Rogers et al., 2013). We have found that there were fewer emigrated NCCs in ethanol treated embryos. Laminin and adhesion molecules play important roles in the EMT process during neural crest generation (Theveneau et al., 2007). We determined that ethanol up-regulated Laminin and N-Cadherin

expression in the dorsal neuroepithelium, which inhibited the neural crest progenitor cells conversion into migratory NCCs (Figure 6). Furthermore, Cadherin 6B proteolysis has been deemed crucial for ensuring proper cranial neural crest EMT (Padmanabhan and Taneyhill, 2015). It also implies that the ethanol exposure-repressed EMT process might be achieved through altering the expressions of adherent molecules. Transcription factors and cytokines, Msx1 and BMP4, regulate the neural crest cells EMT process. Furthermore, BMP4 plays an important role in the induction, migration and differentiation of NCCs - as well as during alveolar bone formation (Jia et al., 2016). Slug and FoxD3 are normally involved in regulating genes that control cell adhesions, which have direct impact on cell motility. Taken together, these pieces of data imply that ethanol exposure can negatively alter gene expressions involved in EMT and adhesion molecules, thereby inhibiting cranial NNC production.

We have summarized the overall results our study in Figure 10. Firstly, the diagram shows ethanol inhibiting Ap-2α^{+} NCC proliferation and enhancing apoptosis. Secondly, ethanol exposure repressed BMP4 and Msx1 signaling which in turn down-regulated Slug and FoxD3 expression. This also up-regulated N-Cadherin and Cadherin 6B expression while Cadherin 7 expression was down-regulated. Together, these changes interfered with neural crest EMT process. Thirdly, ethanol exposure inhibited HNK-1^{+} NCC migration and differentiation. We propose that the combination of these aberrant events (especially the EMT process) in the cranial NCCs resulted in the abnormal development of the craniofacial bones.

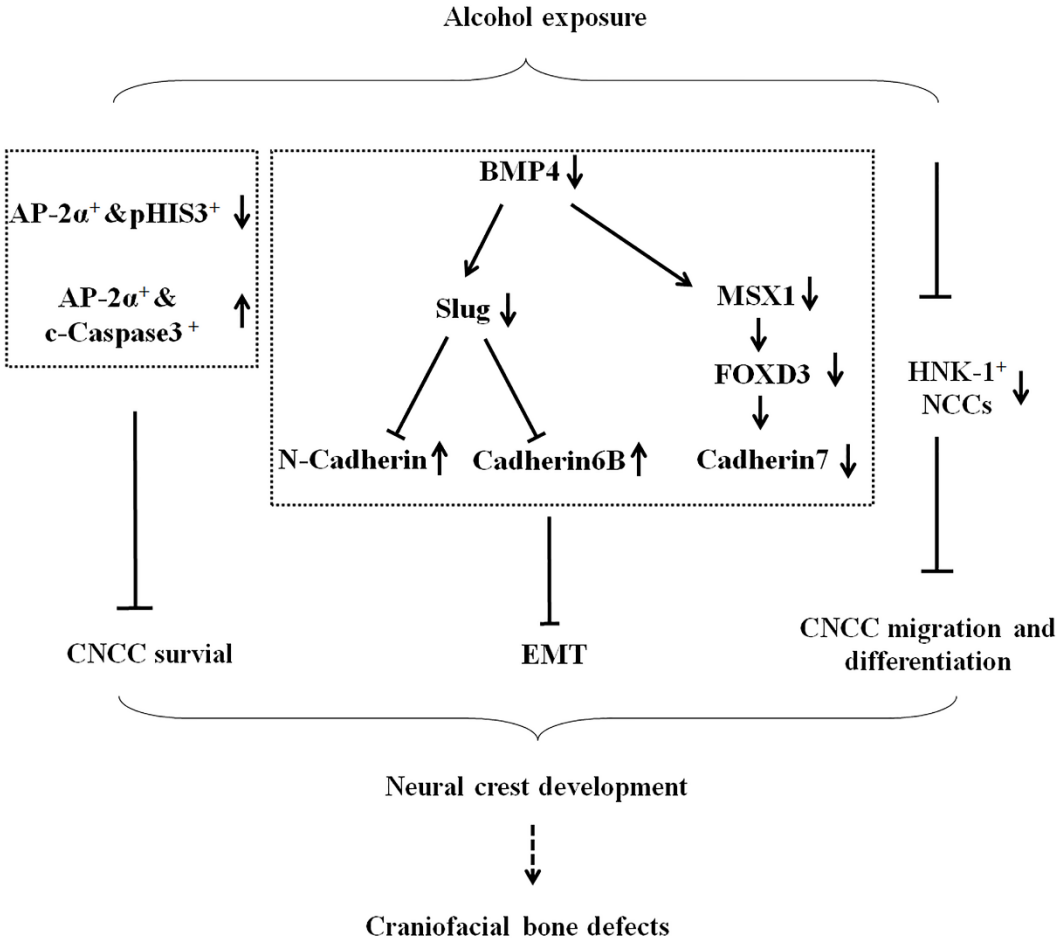

Fig 10. Hypothetical model illustrating how ethanol exposure induces cranial neural crest cell dysplasia.

Acknowledgements

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Gene(chick)	in-situ hybridization probe primer
MSX1	GCGAGGAGGAGAGCGACAAAC ATTAACCCTCACTAAAGGGGATGAAGGGAGCGGAGAAGTC
BMP4	TTATAAAAGCTTGCGGCCGCAGAATATATGTTTGGGCTGCGAAGGC GCTCTAGAAATTAACCCTCACTAAAGGGCGTGGTTGGTGGAGTTGAG
FOXD3	CTCATGATGCAGGGCTTCG CCCGTTCGTTCGCTTATTTCG
Cadherin6B	CAAGTGGAAGATGTAGATGAACCCC ATTAACCCTCACTAAAGGTTCTGGCACAATGTCTCTGCG

Supplementary Fig 1. Show sets of primers used to generate antisense probes for the in situ hybridization studies.

BMP4	GGGCTTCCACCGGATAAACA ACATCAAAGGTCTCCCAGCG
MSX1	CTGCATTCGGCATCTCCTTCC CGGCTCGGCCCTATGTAA
FOXD3	TGCAGTACCCCTACATCCCG CTGGGCTCGGATTTCACGAT
Slug	TCCAGACCCTGGCTACTTCA CGAGTGGGTCTGCAGATGAG
Laminin	TGACCTTCAAGACCTTCCGC GATTACGTCGTCAATGCGGC
N-Cadherin	CTGGGGACATTGGGGACTTC ATAGTCTTGCTCACCACCGC
Cadherin6B	TACAGCATCACTGAGGGGGA CCACGTGAGTATTGGTGGCT
Cadherin7	GGGCTCAGACCCACTCTATG CCAGCCTCTTTGTAGCGTGA
GAPDH	TCAAATGGGCAGATGCAGGT AGCTGAGGGAGCTGAGATGA

Supplementary Fig 2. Sets of primers used in the RT-qPCR analysis.

1
2